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Mutation of a Single Envelope N-Linked Glycosylation Site Enhances the Pathogenicity of Bovine Leukemia Virus

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ABSTRACT

Viruses have coevolved with their host to ensure efficient replication and transmission without inducing excessive pathogenicity that would indirectly impair their persistence. This is exemplified by the bovine leukemia virus (BLV) system in which lymphoproliferative disorders develop in ruminants after latency periods of several years. In principle, the equilibrium reached between the virus and its host could be disrupted by emergence of more pathogenic strains. Intriguingly but fortunately, such a hyperpathogenic BLV strain was never observed in the field or designed *in vitro*. In this study, we sought to understand the role of envelope N-linked glycosylation with the hypothesis that this posttranslational modification could either favor BLV infection by allowing viral entry or allow immune escape by using glycans as a shield. Using reverse genetics of an infectious molecular provirus, we identified a N-linked envelope glycosylation site (N230) that limits viral replication and pathogenicity. Indeed, mutation N230E unexpectedly leads to enhanced fusogenicity and protein stability.

IMPORTANCE

Infection by retroviruses requires the interaction of the viral envelope protein (SU) with a membrane-associated receptor allowing fusion and release of the viral genomic RNA into the cell. We show that N-linked glycosylation of the bovine leukemia virus (BLV) SU protein is, as expected, essential for cell infection *in vitro*. Consistently, mutation of all glycosylation sites of a BLV provirus destroys infectivity *in vivo*. However, single mutations do not significantly modify replication *in vivo*. Instead, a particular mutation at SU codon 230 increases replication and accelerates pathogenesis. This unexpected observation has important consequences in terms of disease control and managing.

Bovine leukemia virus (BLV) is a deltaretrovirus closely related to the human T-lymphotropic virus type 1 (HTLV-1) (1). Both viruses have a similar genomic organization and infect cells of the hematopoietic system. While BLV replicates in B lymphocytes, HTLV-1 infects $CD4^+$ T, $CD8^+$ T, and dendritic cells. HTLV-1 induces a neuroinflammatory disease (HTLV-associated myelopathy/tropical spastic paraparesis [HAM/TSP]) and a T-cell leukemia/lymphoma (adult T cell leukemia/lymphoma [ATLL]). There is no satisfactory treatment for HAM/TSP, and the prognosis for ATLL is still poor despite improved therapies (2). BLV is responsible for major economic losses in cattle due to export limitations, carcass condemnations, and reduction in milk production. BLV infection also correlates with a significant morbidity resulting from opportunistic infections and a decrease in longevity due to tumor development (3). In a proportion (i.e., about one third) of infected cattle, BLV induces a lymphoproliferative disease called persistent lymphocytosis. After a latency period of several years, BLV infection also leads to leukemia and/or lymphoma in a minority (5%) of the infected animals. However, the majority of BLV carriers remain clinically healthy and acts as asymptomatic carriers for viral spread (4). Besides its natural hosts (i.e., cattle, zebu, and water buffalo), BLV can be experimentally transmitted to sheep and goats, where leukemia/lymphoma develops after shorter latency periods (5–7).

Retroviral envelope glycoproteins play an important role in the viral life cycle: they contain the recognition site required for entry and mediate cell fusion (8). The BLV envelope is composed of two glycoproteins: a surface (SU) protein, Gp51, and a transmembrane (TM) protein, Gp30, derived from the proteolytic cleavage of a common precursor (gpr72) encoded by the *env* gene (9–11). The SU protein is N-glycosylated in the rough endoplasmic reticulum by covalent attachment of oligosaccharide chains (12). Although the role of BLV SU-linked glycans is currently unknown, it is expected that N-glycosylation is required for protein folding, stability, or solubility (13). Furthermore, N-glycans are also likely involved in transport of BLV envelope proteins to the cell membrane, binding to cellular receptors and cell-to-cell fusion (14–18) similarly to glycans of the human immunodeficiency virus (HIV) envelope glycoprotein that modulate fusogenicity and viral infectivity (19). By shielding viral epitopes, SU-associated N-glycans also exert an important escape function from the host immune response (14, 17). Being of a lesser interest, simian immunodeficiency virus (SIV) mutants lacking specific N-linked glycans dem-

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onstrate a markedly increased antibody binding to gp120 envelope, suggesting a role of glycosylation in immune escape (20).

The aim of the present study was to investigate the role of BLV envelope carbohydrates in infectivity and pathogenicity. We first show that N-glycans of the BLV SU are, as expected, required for cell-to-cell infection. Individual substitutions of the 8 N-linked glycosylation sites showed only modest effects, with the marked exception of N230E. This BLV mutant unexpectedly replicated faster and was more pathogenic than the parental isogenic strain. To our knowledge, this is the very first time that a hyperpathogenic deltaretrovirus is created by a single amino acid mutation.

MATERIALS AND METHODS

Site-directed mutagenesis. Vectors for envelope mutants were constructed by site-directed mutagenesis using the pSGenv plasmid vector (21, 22). The PCR was performed according to the supplier's protocol described in the QuikChange Multi site-directed mutagenesis kit (Stratagene) using primers carrying the asparagine (N) to glutamic acid (E) codon mutation. Briefly, 100 ng of plasmid was amplified in the presence of 1 μl of a deoxynucleoside triphosphate (dNTP) mix, 0.5 μl of QuickSolution, 2.5 μl of QuikChange Multi reaction buffer, 1 μl of QuikChange Multi enzyme blend, and 100 ng of each primer/μl. After denaturation for 1 min at 95°C, 30 cycles of PCR were performed: 1 min denaturation at 95°C, 1 min annealing at 55°C, and 16 min of elongation at 65°C. The PCR was performed in a Veriti 96-well thermal cycle apparatus (Applied Biosystems). After amplification, the samples were digested with 10 U of restriction enzyme DpnI for 1 h at 37°C to remove the parental DNA strand. DNA was then amplified by bacterial transformation in Ultracompetent cells (Stratagene). The mutated proviruses were constructed by using a QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the supplier's recommendations. After DNA minipreparation (Qiagen), the mutated plasmids and proviruses were sequenced to confirm the presence of the mutation.

Cells lines. HeLa (human uterine carcinoma), HEK293T (human embryonic kidney), and COS-7 (simian virus 40-transformed *Cercopithecus aethiops* kidney) cells obtained from the American Type Culture Collection were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 μg of penicillin-streptomycin/ml. The feline kidney CC81 cell line was cultivated in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin. These cell lines were maintained in a humidified incubator at 37°C in a 5 to 95% CO_2-air atmosphere. HeLa and COS-7 cells were transfected with SU expression vectors or proviral plasmids using Mirus Trans IT-LT1 reagent (Mirus Bio), as recommended by the manufacturer. HEK293T cells were transfected after calcium phosphate precipitation.

Syncytium formation assay. To screen for the formation of multinucleated cells in the presence of glycosylation inhibitors and lectins, HEK293T cells plated on 10-mm-diameter petri dishes were transfected with a plasmid containing a cloned BLV provirus (pBLV344) and treated for 16 h with lectins or N-linked glycosylation inhibitors. The glycosylation inhibitors tunicamycin, deoxynojirimycin, monensin, and deoxymannojirimycin were purchased from EMD Biosciences, while swainsonin was obtained from Sigma-Aldrich. The two lectins used *Urtica dioica* lectin (UDA) and *Hippeastrum* hybrid agglutinin lectin (HHA) were obtained from EY Laboratories, Inc. After 5 days of coculture with the CC81 cell line at a ratio of 1 to 5, the cells were colored with May-Grünwald-Giemsa reagent. The number of syncytia with more than 10 nuclei was then scored by visualization under an optical microscope. For the fusion assay with the various glycosylation mutants, COS-7 cells were transfected with pBLV344 or with envelope-expression vectors. At 48 h posttransfection, the cells were cocultivated during 1 day with CC81 at a 1:5 proportion and then visualized as described above.

Western blotting. HeLa cells were transfected with expression vectors for wild-type (pSGenv) or mutant (N67E, N129E, N203E, N230E, N251E, N256E, N271E, and N287E) SU. For protein stability assays, cells were incubated with 50 μM cycloheximide (Sigma-Aldrich). After 48 h, the cells were washed twice in cold phosphate-buffered saline (PBS) and lysed for 20 min at 4°C with high-salt lysis buffer (LB400; 0.1 M Tris-HCl [pH 7.2], 400 mM NaCl, 0.5% NP-40) supplemented with 1 mM dithiothreitol and antiprotease cocktail (Complete; Roche Diagnostics). After quantification by the Bradford method (Bio-Rad), a 20-μg portion of the proteins was migrated onto a 12% SDS-polyacrylamide gel for 1 h at room temperature and transferred onto a nitrocellulose membrane (GE Healthcare). After blocking with Tris-buffered saline (TBS)–0.1% Tween 20 containing 4% of nonfat dry milk, the membrane was incubated during 1 h at room temperature with primary antibodies. Antibody concentrations were provided by the manufacturer and tested experimentally: anti-SU (BLV_2; dilution, 1:2,000; VMRD), C23 (nucleolin [NCL]; dilution, 1:1,000; Santa Cruz Biotechnology). The nitrocellulose membrane was washed three times for 5 min with TBS–0.1% Tween 20 and then incubated for 1 h with a horseradish peroxidase-labeled goat anti-mouse secondary antibody (dilution 1:5,000; Santa Cruz Biotechnology). After three washes, the membrane was revealed by chemiluminescence (ECL Plus Western blotting detection kit; GE Healthcare).

Ethics statements. Animal experiments were conducted in accordance with national (Royal Decree on the Protection of Experimental Animals) and international (European Commission) guidelines for animal care and use described in the ULg manual for use and care of experimental animals. Handling of sheep and experimental procedures were reviewed and approved by ULg's Institutional Committee for Care and Use of Experimental Animals under protocol 1515.

Infection of sheep with recombinant proviruses. A total of 30 sheep were maintained under restricted conditions at the National Veterinary Research Institute in Pulawy (Poland) and the CEPA of GxABT Agricultural University in Gembloux (Belgium). Animals were maintained in L2-restricted containment in agreement with national and European regulations under registration number LA1900600. Sheep were inoculated with BLV proviruses cloned into a bacterial vector, as previously described (23, 24).

Titration of anti-BLV antibodies. Venous blood was collected by jugular venipuncture and mixed with 0.3% (wt/vol) EDTA used as an anticoagulant. Plasma was separated from blood after centrifugation at 1,880 × *g* for 25 min. The plasma samples were then stored at −20°C or directly used for analysis. The presence of anti-BLV antibodies was determined using a competitive enzyme-linked immunosorbent assay (ELISA; IDEXX Leukosis Blocking Ab Test) according to the manufacturer's recommendations.

Isolation of PBMCs. Leukocyte and lymphocyte counts were determined using an automated cell counter (MS 4-5 Vet; Melet Schloesing Laboratories). Peripheral blood mononuclear cells (PBMCs) were separated by Percoll density gradient centrifugation (GE Healthcare) and washed twice with PBS–0.075% EDTA and at least three times with PBS alone to eliminate platelets. After estimation of their viability by trypan blue dye exclusion, the PBMCs were either cryopreserved in dimethyl sulfoxide (Sigma-Aldrich)-FBS (10/90 [vol/vol]) or directly used for analysis.

Quantification of the proviral load. Genomic DNA was extracted from Percoll gradient-purified PBMCs using a DNeasy blood and tissue kit as recommended by the manufacturer (Qiagen). A total of 100 ng of genomic DNA was used for real-time PCR amplification of BLV proviral sequences. A segment corresponding to the *pol* gene (nucleotides 3994 to 4060, according to the BLV GenBank entry with accession number K02120) was amplified with a pair of primers (5′-GAAACTCCAGAGCA ATGGCATAA-3′ and 5′-GGTTCGGCCATCGAGACA-3′ at a 900 nM final concentration). In order to correct for differences in DNA concentrations and amplification efficiencies between samples, the 18S rRNA genes were quantified in parallel using the primers 5′-TTGGATAACTGT

GGTAATTCTAGAAGCTAA-3′ and 5′-CGGGTTGGTTTTGATCTGAT AAAT-3′. DNA was amplified by real-time quantitative PCR in a StepOne Apparatus (Applied Biosystems) using MESA green master mix (Eurogentec). The thermal protocol included a 95°C denaturation step for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s, and then a melting curve. The number of viral copies was obtained by PCR using a standard curve of serial dilutions of plasmid pBLV344. The number of viral copies was normalized to a standard curve performed with the 18S cellular gene (BLV/18S). The number of viral copies per cell was then obtained by multiplying the BLV/18S ratio by the average value of the number of 18S copies per cell. Each of the viral loads for each animal at each time point were determined by three independent quantitative PCR amplifications of DNAs extracted independently.

PCR amplification and sequencing. After DNA extraction of a tumor biopsy specimen (25 mg), a segment corresponding to the *env* gene (nucleotides 4791 to 5764, according to the BLV GenBank entry accession number K02120) was amplified by PCR in a Veriti 96-well thermal cycle apparatus (Applied Biosystems) using the primers 5′-ACTGACAGGGC TAGGCC-3′ and 5′-GGTTCCCTGGCGTTT-3′. Briefly, in a final volume of 50 μl, 500 ng of DNA was amplified in the presence of 1 μl of dNTP mixture (2 mM), 4 μl of $MgCl_2$ (25 mM), 1 μl of *Taq* polymerase (5 U/μl), 5 μl of 10× *Taq* buffer, and 2.5 μl of each primer (10 μM). The thermal protocol included an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and then a final extension step at 72°C for 5 min. After amplification, the PCR product was visualized on a 1% agarose TAE gel. The PCR fragment was then purified using the QIAquick gel extraction kit (Qiagen) and sequenced using the primer 5′-ACTGACAGGGCTAGGCC-3′.

RESULTS

BLV-induced cell-to-cell fusion requires glycosylation. To evaluate the role of glycans in viral infection, we first used a strategy based on pharmacological inhibition of glycosylation. Five inhibitors were tested for their ability to interfere with virus-induced cell-to-cell fusion: deoxynojirimycin, an antibiotic produced by *Bacillus* sp., inhibiting the early glycosylation steps by acting on glucosidase I; deoxymannojirimycin and swainsonine blocking mannosidases I and II in the Golgi apparatus; monensin, an ionophore that inhibits intracellular transport from the ER to the Golgi apparatus; and tunicamycin, an antibiotic produced by *Streptomyces lysosuperficus*, interfering with the first step of glycosylation (the addition of *N*-acetylglucosamine to dolichol phosphate). Because cell-free infection is very inefficient due to high instability of the BLV virion (25), a model based on cell-to-cell infection was tested. HEK293T cells were first transfected with a plasmid containing a cloned BLV provirus (pBLV344), cultivated for 16 h with the above-mentioned inhibitors, and finally mixed with CC81 indicator cells. The number of multinucleated cells (syncytia) induced by the BLV provirus was arbitrarily set to 100%. Figure 1A shows that, regardless of the glycosylation inhibitor used, the ability of the virus to form syncytia was strongly affected. Under the conditions used, the inhibitors did not significantly affect cell viability or protein expression levels, as determined by an MTS assay and Western blotting, respectively (data not shown). Since these inhibitors likely have many off-target effects, we further used a lectin-based approach to assess the role of glycans in BLV infection. Indeed, lectins directly bind to glycans without affecting the molecular mechanisms of intracellular glycosylation. Two different concentrations of lectins (1 and 10 μg/ml) were tested for their ability to affect cell fusion. Figure 1B shows that syncytium formation is drastically reduced under all conditions tested. Together, these results indicate that glycosylation is involved in cell-to-cell infection by BLV, confirming and extending reports from the literature (14, 15, 26–28).

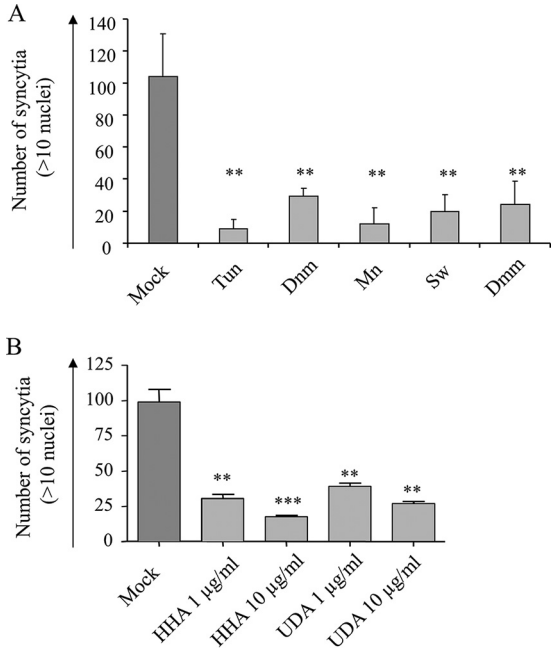


FIG 1 Effect of glycosylation inhibitors and lectins on syncytium formation. (A) HEK293T cells were transfected with a plasmid containing a BLV provirus (pBLV344) and treated with glycosylation inhibitors: 2.4 μM tunicamycin (Tun), 3 mM deoxynojirimycin (Dnm), 1 μM monensin (Mn), 60 μM swainsonin (Sw), or 5 mM deoxymannojirimycin (Dmm). At 16 h after treatment, HEK293T cells were cocultivated with indicator cells (CC81) in a 1:5 proportion, and the numbers of syncytia containing more than 10 nuclei were scored after 5 days. The graph represents average numbers (± the standard deviations [SD]) of three independent experiments. (B) HEK293T cells were transfected with plasmid pBLV344. At 48 h after transfection, cells were cocultivated with CC81 cells (ratio 1:5) and placed in contact with lectins (HHA and UDA at 1 and 10 μg/ml) for 5 days. The cells were fixed with methanol and stained with Giemsa to determine the numbers of syncytia by light microscopy (magnification, ×100). **, $P < 0.01$; ***, $P < 0.001$ (statistical significance according to the Student *t* test performed with data of three independent experiments).

Mutation of the N-glycosylation site N230E confers improved fusion capacity. To get a deeper insight into the role of glycans in BLV infection, we next focused on potential N-glycosylation sites present on the SU envelope protein (Fig. 2A). Therefore, the eight consensus N-glycosylation sequences (NXS/T) were modified by site-directed mutagenesis to convert the asparagine codon (N) into glutamic acid (E), yielding N-to-E mutations at positions 67, 129, 203, 230, 251, 256, 271, and 287 (the numbers refer to the amino acid position in the envelope sequence). After cloning into the pSG5 vector, expression of the mutated envelopes was measured on Western blots (Fig. 2B). Most mutants were expressed at the same level than the wild-type control. Interestingly, but not unexpectedly, the electrophoretic mobility of four mutants (N129E, N203E, N230E, and N251E) was increased compared to wild-type SU and in a nonuniform manner. A difference in apparent molecular mass of about 3 to 4 kDa suggests that these sites are involved in carbohydrate binding (29).

We next ran syncytium assays to determine the role of individual N-glycosylation sites in cell fusion. At 48 h after the transfection of mutant expression vectors, the cells were cocultivated with

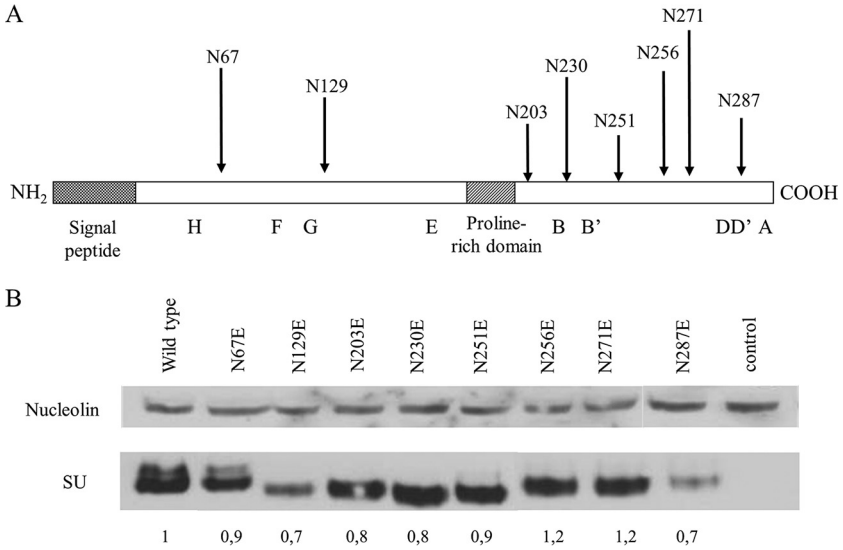


FIG 2 Schematic representation of N-glycosylation sites of the BLV SU protein and expression of SU glycosylation mutants. (A) The eight potential N-glycosylation sites N67, N129, N203, N230, N251, N256, N271, and N287 (where N is Asn followed by a number referring to the amino acid position) are indicated by arrows. Monoclonal antibodies directed toward conformational (F, G, and H) and sequential epitopes (A, B, B′, D, D′, and E) have been generated. A series of N-to-E (asparagine-to-glutamic acid) mutants were generated by site-directed mutagenesis. The grid box corresponds to the signal peptide sequence, and the shaded box is the proline-rich domain. (B) HeLa cells were transfected with a vector encoding the wild-type SU, the different glycosylation mutants, or an empty vector (control). At 48 h after transfection, the cells were lysed and analyzed by Western blotting with antibodies directed against the D and D′ epitopes of SU. Nucleolin was used as a loading control. Numbers refer to quantification of bands intensities of the different envelope Western blot signals using the Bio 1D program (Fusion F; Vilber Lourmat) and expressed as the ratio of SU/nucleolin intensity values.

indicator cells, and the numbers of syncytia with more than 10 nuclei were scored. Figure 3 reports the number of syncytia induced by each SU mutant normalized to the wild-type level arbitrarily set to 100%. SU glycoproteins with a single N-to-E mutation showed no (N67E) or a slight (N129E, N203E, N251E, N256E, N271E, and N287E) reduction in fusion capacity. In contrast, mutant N230E yielded a very significant increase of almost 4-fold in syncytium formation compared to wild-type levels. To extend this observation, we tested two other mutations of alanine and glutamine at protein position 230, namely, N230A and N230Q. The three mutants—E, A, and Q—had a remarkably increased ability to produce additional syncytia (Fig. 3B and C), thereby excluding a role of the codon substitution *per se*. Together, these data demonstrate that mutation of a single residue at position 230 has drastic effects on the ability of SU to form syncytia.

The half-life of the N230E envelope protein is higher than that of the wild-type envelope protein. Although the improved fusion capacity of N230E was not due to an increased expression at the cell membrane (data not shown), it remained possible that the stability of this mutant was enhanced. To answer the question, cells expressing wild-type and N230E proteins were incubated with cycloheximide to block protein synthesis. Serial Western blot analyses extending up to 10 h in the presence of the drug showed a gradual decrease in concentration of both SU proteins, indicating their progressive degradation (Fig. 4A). Quantification of luminescence intensities confirmed that N230E was indeed statistically less degraded than wild-type SU (Fig. 4B). We have thus identified a mutant of BLV SU (N230E) with an improved ability to form syncytia associated with a higher protein half-life.

All SU glycosylation mutants are infectious. Reverse genetics applied to the BLV system offers a unique opportunity to measure the impact of specific mutations on infectivity, viral replication, and pathogenesis. The study is based on inoculation of sheep with a BLV proviral clone (pBLV344). The consensus N-glycosylation sites were modified by site-directed mutagenesis creating eight isogenic proviruses carrying single substitutions: N67E, N129E, N203E, N230E, N251E, N256E, N271E, and N287E. Each N-glycosylation deficient mutant was inoculated into three sheep in order to assess its infectious potential. Figure 5 shows that all mutants were infectious, as assessed by the continuous presence of anti-BLV antibodies in the plasma (the classical method to identify BLV carriers). As a control, sentinel sheep maintained in similar conditions remained uninfected. Of note, mutation of all eight sites simultaneously abrogated infectivity, as indicated by the absence of an anti-BLV humoral response (data not shown).

Next, the anti-BLV antibody titers in two groups of three sheep inoculated either with wild-type or with N230E proviruses were compared. However, no statistically significant differences were observed between these two groups (data not shown). It thus appears that all single N-to-E mutants are infectious, as indicated by the persistence of antiviral antibodies.

N230E replicates faster that wild-type BLV. We next aimed at measuring the replication rate of the eight mutants, and more particularly that of mutant N230E. Therefore, the proviral load was assessed by real-time PCR in groups of three sheep, each infected by one of the eight individual mutants for a total of 24 sheep. Figure 6A shows the mean proviral load (expressed in copy number per PBMC) of all sheep at different times postinfection (at seroconversion and at 3, 6, and 9 months after seroconversion). The mean proviral loads of sheep infected with mutants N129E, N203E, N251E, N271E, and N287E were close to that for the wild type. There was a slight non-statistically significant trend of reduced proviral loads in N67E- and N256E-infected sheep. In con-

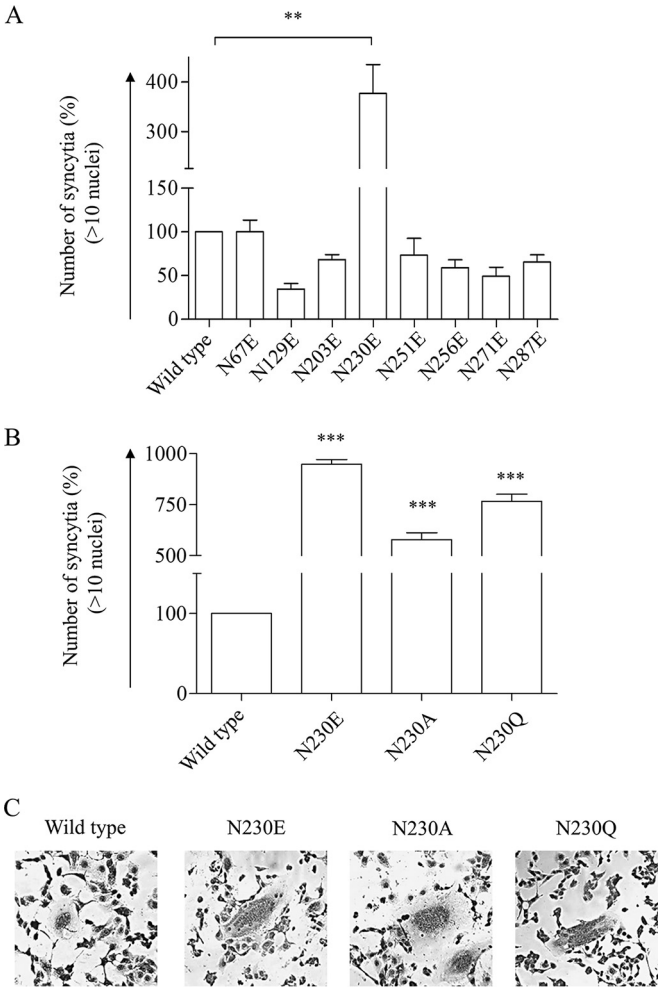


FIG 3 Cell fusion capacity of the N-glycosylation mutants. (A) COS cells were transfected with vectors encoding the wild-type SU or the different glycosylation mutants. At 48 h after transfection, cells were cocultivated with CC81 indicator cells in a 1:5 proportion, and the numbers of syncytia were counted after 1 day of coculture. The results are expressed as ratios between numbers of syncytia generated by mutant and wild-type SU (as a percentage). The data in means ± the SD result from three independent experiments. (B) COS cells were transfected with vectors encoding the wild-type SU or different N230 glycosylation mutants containing glutamic acid (E), alanine (A), or glutamine (Q), instead of asparagine (N). The data in means ± the SD result from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (significance of the results according to Student *t* test). (C) Representative photographs of wild-type- and N230-induced syncytia. Cells were fixed with methanol, stained with Giemsa, and visualized by light microscopy (magnification, ×10).

trast, the proviral load kinetics at 3, 6, and 9 months after seroconversion were significantly increased in the three sheep infected with mutant N230E (Fig. 6A and B). Consistent with higher proviral loads, there was a trend toward accelerated pathogenesis in sheep inoculated with N230E (Fig. 6C): one developed leukemia less than a year after seroconversion, and the two others developed lymphoma within 15 months after seroconversion (Fig. 6C). As a control, PCR amplification and sequencing demonstrated that the N230E mutation did not revert in the tumor cells (Fig. 6D). This impressively short latency period is observed only in splenectomized sheep with impaired immune response (30).

DISCUSSION

In this report, we investigated the role of eight potential N-glycosylation sites of the BLV SU protein and observed their impact on protein stability, induction of syncytia, virus infectivity, and induction of leukemia/lymphoma. Use of pharmacological inhibitors and lectins suggested that, as expected, glycosylation is involved in cell fusion, confirming and expanding previous reports (26, 31, 32) (Fig. 1). Lectins have also been shown to interact directly with carbohydrates and inhibit infection by HIV-1 (33) and HTLV-1 (34). In HIV-1, carbohydrates are involved in binding of gp120 (SU) to cell receptors (CD4, CCR5, and CXCR4) on $CD4^+$ cells (19, 35–37). Although the glycosidic structure of BLV SU is unknown, glycanic chains contain at least galactose, mannose, and glucosamine, which are possibly involved in receptor binding (38, 39). The reduced ability of the virus to fuse in the presence of the two lectins used, HHA (a mannose-specific lectin) and UDA (an *N*-acetylglucosamine-specific lectin), as observed in the presence of glycosylation inhibitors, reinforces the idea that carbohydrates present on BLV SU are implicated in the attachment of the virus to its cellular receptors, as shown for HIV-1 (40, 41).

The major purpose of our study was to investigate the impact of mutations affecting the BLV consensus N-glycosylation sites of the SU protein. It is noteworthy that the N-glycosylation consensus sequences are perfectly conserved among all known BLV strains (42), suggesting their biological relevance. Comparative analysis of SU migration rate in polyacrylamide gels suggested that four of the eight N-glycosylation sites (N129, N203, N230, and N251) might indeed be involved in carbohydrate binding (Fig. 2B). Although glycan interaction with other consensus sequences cannot be formally excluded, a difference of about 3 to 4 kDa is generally associated with carbohydrates bound to a single glycosylation site (43, 44). Among the four mutants, the N230E protein had the fastest electrophoretic mobility, suggesting a significant contribution of the N230 glycan in the overall glycan mass in the wild-type protein. Such a contribution could reflect peculiarities in glycan structure or composition (e.g., high mannose content and hybrid or complex backbones) (45). To address these issues, further experiments using, for example, mass spectrometry will be required.

A first rather unexpected result was that most mutations did not completely abolish cell fusion (Fig. 3), although N129E was slightly impaired. It is possible that the fusion assay used, which best recapitulates the natural cell-to-cell infection mode, lacks sensitivity. Consistently, however, all mutants remained infectious *in vivo*, indicating that individual mutation of SU N-glycosylation sites does not abolish infectivity (Fig. 5A). Of note, concomitant mutation of N129, N203, N230, and N251 preserved cell fusion capacity. In contrast, mutation of all eight potential sites generated a defective envelope precursor (gpr72) unable to induce syncytia (data not shown). In the related HTLV-1 system, N-linked glycosylation sites have also been involved in cell-cell fusion (26).

A most interesting observation was the hyperfusogenic phenotype exhibited by the N230E mutant (Fig. 3). This conclusion, generated from a standard fusion assay, was confirmed by another system based on the use of primary cells (data not shown). Previous reports on HIV and SIV (19, 46, 47) showed that the absence of a single glycosylation site had no dramatic effect on viral repli-

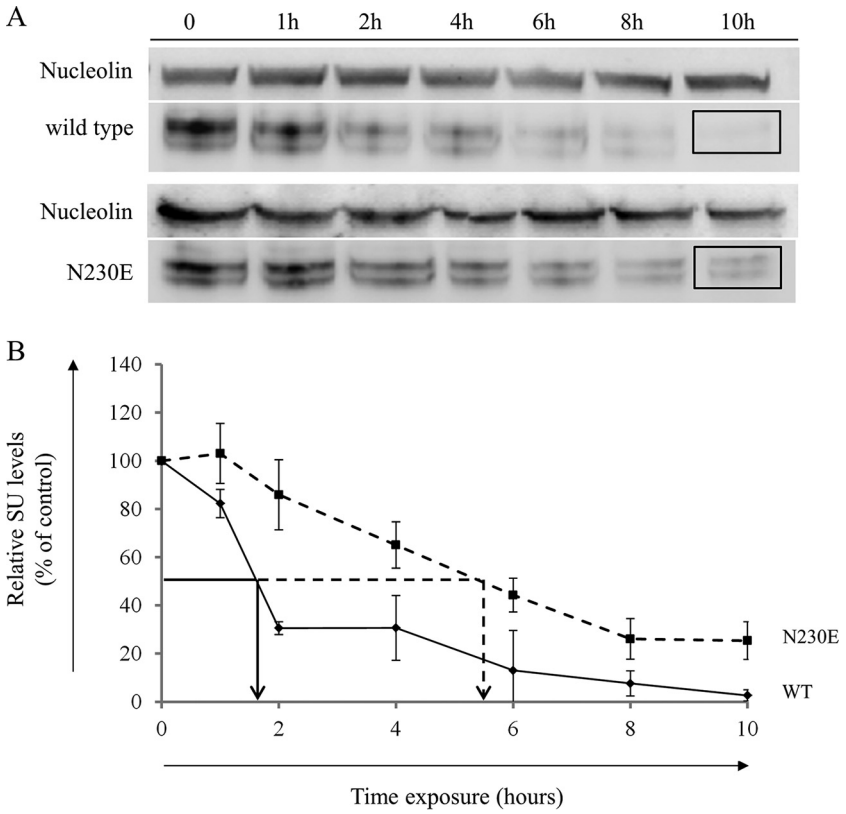


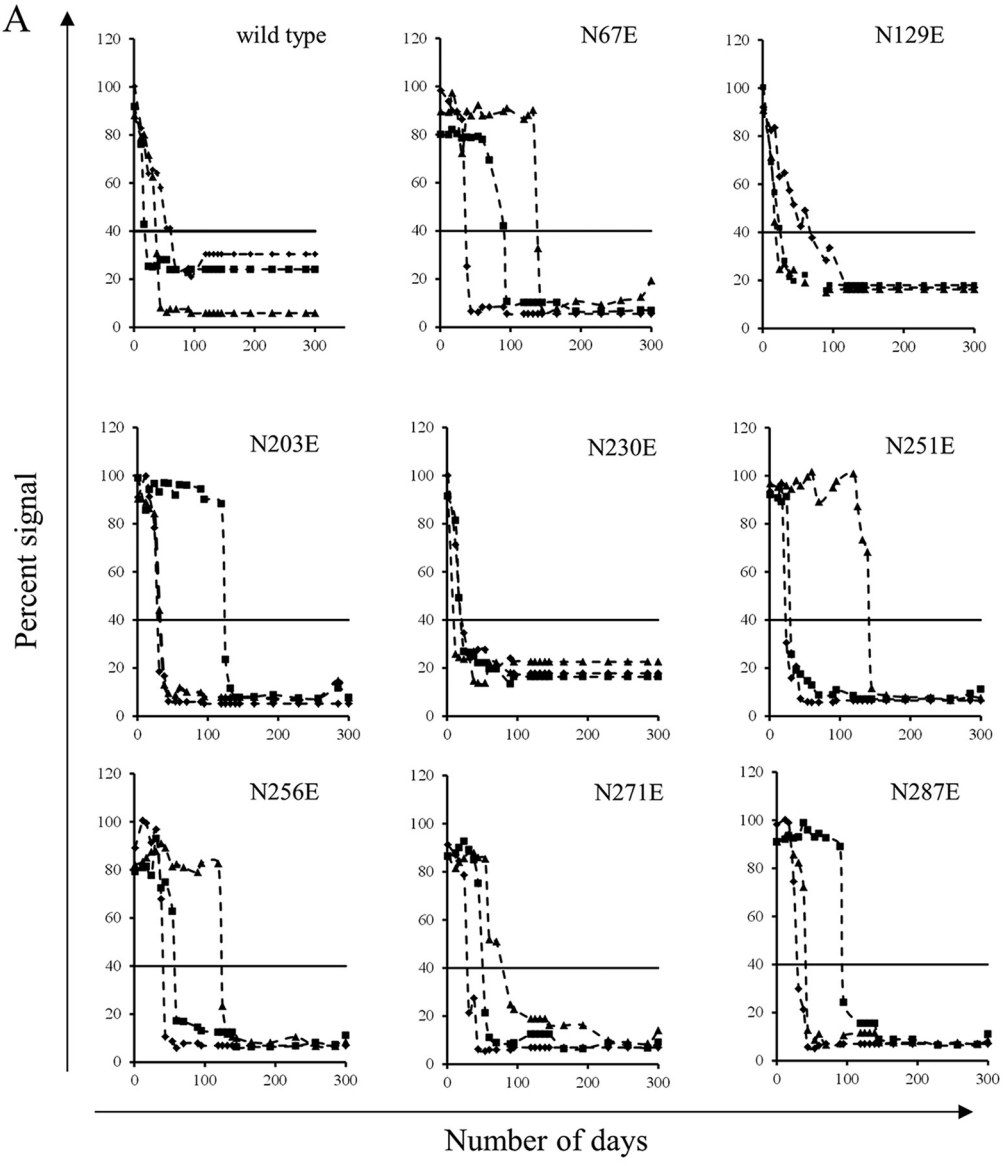
FIG 4 Time course analysis of N230E SU half-life in the presence of cycloheximide. (A) HeLa cells were transfected with vectors encoding the wild-type SU or the N230E glycosylation mutant and cultivated with cycloheximide for 0, 1, 2, 4, 6, 8, and 10 h. The cells were lysed and analyzed by Western blotting with monoclonal antibodies directed against the D and D′ epitopes of the SU protein. Nucleolin was used as a loading control. (B) For quantification, SU luminescence was normalized to that of nucleolin, and the ratios at time zero were arbitrarily set at 100%. The graph represents average numbers (± the SD) of three independent experiments. The solid and dotted arrows represent the half-life values of WT and N230E envelope proteins.

cation. However, hyperfusogenic strains could be obtained by multiple mutations of different glycosylation sites allowing easier accessibility to the receptor and coreceptor if any and leading to increased infectivity (19). The hyperfusogenicity of mutant N230E calls for detailed structural studies dealing with the mechanisms linking the binding of SU to cellular receptors to conformational changes in the TM that insert the fusion peptide into the target cell membrane (27, 48). At this stage, the TM protein presents a "pre-hairpin" conformation as an intermediate that is then stabilized by the assembly of a triple strand coiled coil. All of these conformational changes bring the opposite virus-cell membranes together, resulting in their fusion (48, 49). Possible mechanisms of N230E hyperfusogenicity include (i) a higher affinity of binding to cell receptors (29, 50), (ii) decreased interaction between SU and TM (29, 51), or (iii) an accelerated rate of six-helix bundle formation (14, 29, 52, 53). Nevertheless, our data seem to disfavor a process involving an increased association of N230E protein with the cell membrane (data not shown), which results from a combination of intracellular transport in the donor cell and internalization by the target cell. Without excluding other mechanisms, Fig. 4 clearly shows that the stability of N230E is improved compared to wild-type SU. Decreased proteolytic degradation contributes to hyperfusogenicity of N230E. Hydrophobic cluster analysis profiles show that C-terminal N-glycosylation sites are located right at the end of beta strand structures (Fig. 7). This model predicts that the N-glycosylation sites are carried by easily accessible loops that are likely to readily undergo various conformational changes. Further progress in the puzzling problem of membrane fusogenicity will require experimental studies of the three-dimensional (3D) structures of envelope glycoproteins.

Because SU 230 protein is exposed at the cell surface and quite dynamic, glycans may shield neutralization epitopes essential for SU function. Similarly, oligosaccharides have been shown to limit humoral response to SIV gp120 and NDV fusion protein by hiding B-cell epitopes (20, 44). However, the overall antigenicity of N230E SU was equivalent to that of the wild-type protein. In addition, our results show that a peptide spanning N230 was not targeted by humoral or cytotoxic responses (data not shown). These observations suggest that, rather than hiding a neutralizing epitope, N230 glycans seems rather modulate protein conformation and dynamics, as suggested by hydrophobic cluster analysis profiles (Fig. 7).

Perhaps the most surprising result of this report is that replication of mutant N230E is unusually high, leading to an accelerated onset of BLV-induced malignancy (Fig. 6). Although rarely reported in other viral systems such as in paramyxoviruses (29, 50, 51), hyperpathogenicity due to a single mutation of an N-linked glycosylation site has, to our knowledge, not been observed in deltaretroviruses. In fact, BLV and HTLV-1 share a very particular mode of viral replication that combines cell-to-cell infection and mitotic expansion of provirus-containing lymphocytes. At first glance, it seems obvious that the proviral loads would be increased

A



B

mutant	seroconversion (days)	mutant	seroconversion (days)	mutant	seroconversion (days)
wild type	54	**N67E**	38	**N129E**	54
	17		90		31
	38		130		24
N203E	31	**N230E**	24	**N251E**	24
	125		24		31
	38		12		130
N256E	44	**N271E**	31	**N287E**	31
	60		54		95
	125		90		44

FIG 5 Anti-BLV seroreactivity of sheep inoculated with the N-glycosylation mutants. (A) Kinetics of anti-BLV reactivity in sheep inoculated with wild-type (pBLV344) and mutant (N67E, N129E, N203E, N230E, N251E, N256E, N271E, and N287E) proviruses. The graph shows the evolution over time (in days) of the percentage of signal due to the presence of SU antibodies in the serum to be tested. Forty percent represents the detection threshold below which a sample is considered to be positive as determined by an indirect ELISA (IDEXX, Leukosis Blocking Ab Test). (B) Time difference between inoculation and seroconversion.

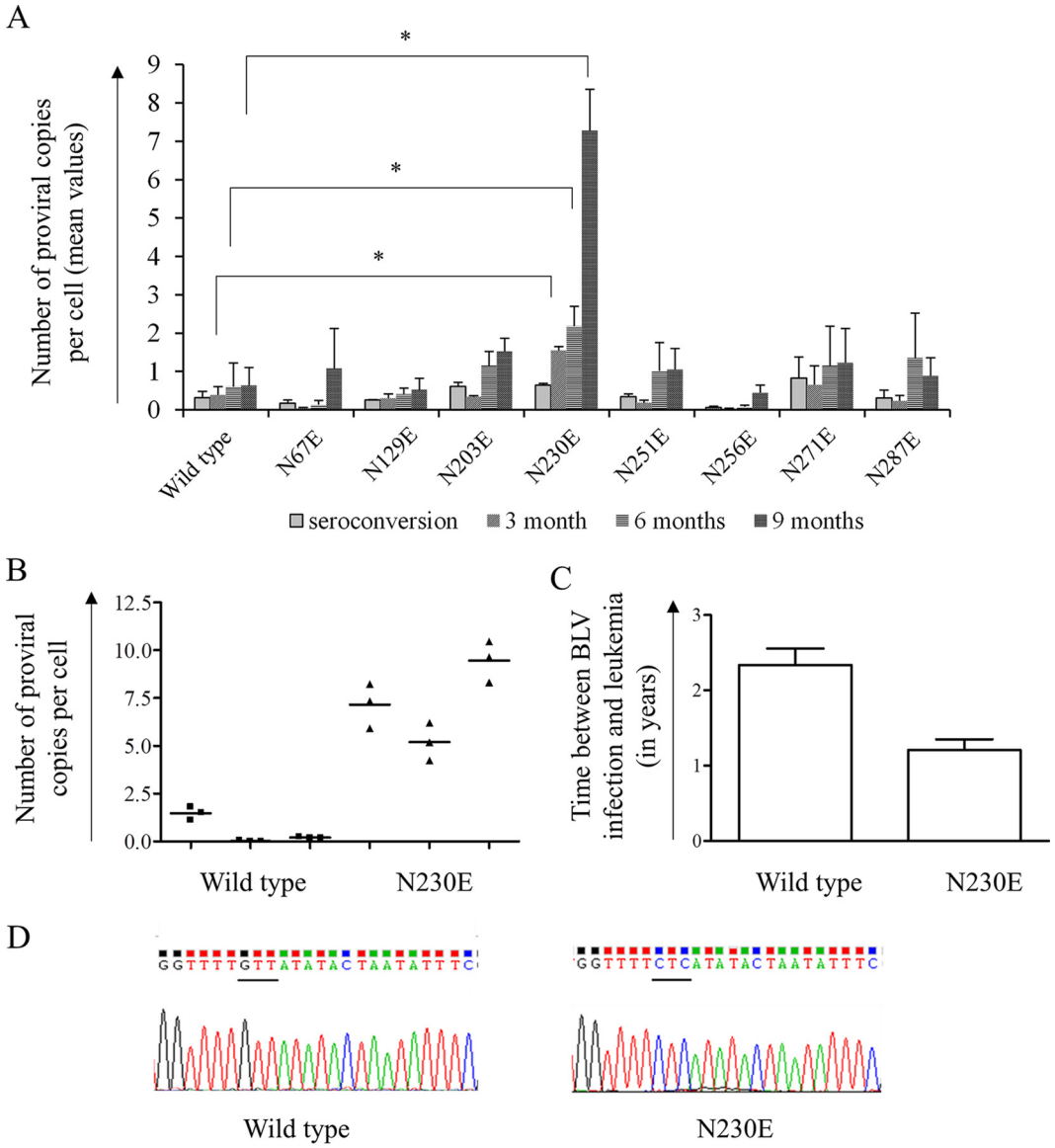


FIG 6 Replication and latency period in sheep infected by N-glycosylation mutants. (A) Proviral loads in sheep infected with wild-type BLV provirus (pBLV344) and SU N-glycosylation mutants. Three sheep were inoculated with wild-type virus (pBLV344) or with glycosylation site mutants (N67E, N129E, N203E, N230E, N251E, N256E, N271E, and N287E). At different times postinfection (at seroconversion and at 3, 6, or 9 months), 100 ng of DNA was extracted from PBMCs, and the number of viral copies was determined by real-time PCR using a standard curve of serial dilutions of plasmid pBLV344. The number of viral copies was normalized to a standard curve performed with the 18S cellular gene (BLV/18S). The number of viral copies per cell was obtained by multiplying the BLV/18S ratio by the average value of the number of 18S copies per cell. *, $P < 0.05$ (statistical significance of the results using a Mann-Whitney nonparametric test made from average proviral copies per cell for the three animals). (B) Proviral loads in sheep infected with the wild-type BLV provirus or mutant N230E at 9 months after seroconversion. A total of 100 ng of DNA was extracted from PBMCs of three sheep infected with wild-type virus (pBLV344) or mutated from N to E at the 230 position (N230E). The number of viral copies was obtained as described above. Average values of three independent qPCRs are shown for three wild-type and three N230E animals. (C) Development of leukemia/lymphoma in sheep infected with mutant N230E. Mean (+ the SD) latency periods (i.e., the time between seroconversion and leukemia/lymphoma) are indicated, as are the leukocyte counts > 40,000/mm^3) in sheep infected with the wild-type BLV provirus ($n = 27$) or with the N-to-E mutant provirus at position 230 ($n = 3$). (D) Sequencing profiles of DNA extracts from PBMCs of leukemic/lymphomatous sheep. The figure shows the presence of the mutation at glycosylation site 230 in DNA extracted from the PBMCs of sheep infected with the corresponding mutated provirus.

if infection of new target cells by mutant N230E is enhanced. However, cell-to-cell transmission can only be significantly detected at the very early stages of infection because of a very active immune response that only tolerates clonal expansion (54, 55). Consistently, a TM mutant impaired in cell fusion (A60V) (56) can replicate at wild-type levels and induce lymphoma/leukemia in sheep, emphasizing the primordial role of clonal replication during chronic infection. The kinetics of proviral loads (Fig. 6) indicates that N230E maintains a selective replicative advantage throughout infection. High-throughput sequencing of proviral insertion sites (24) might provide some tentative explanation for the observed pathogenicity of N230E virus. These data will also determine whether N230E promotes a faster clonal expansion than other viruses instead of promoting viral replication through

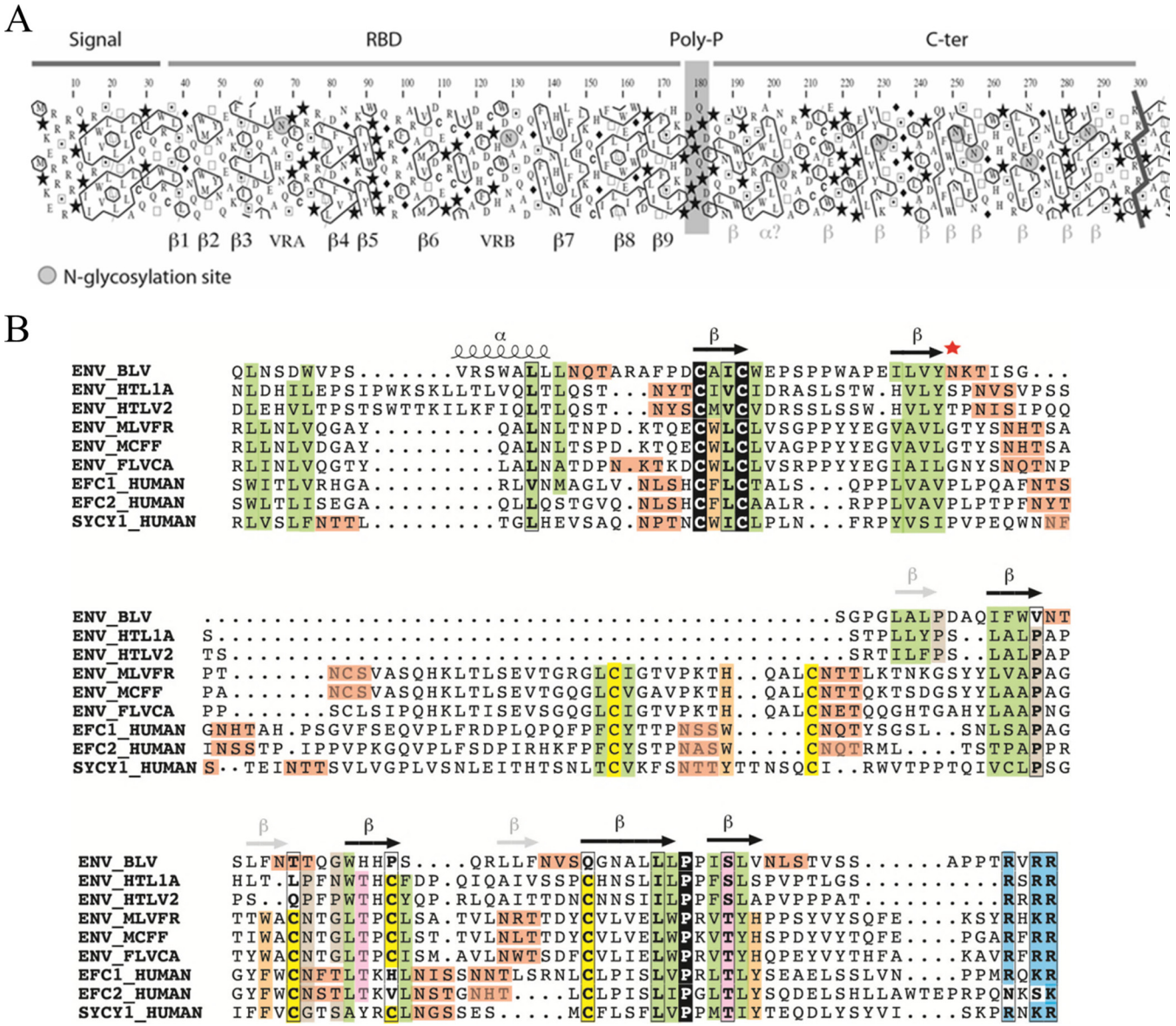


FIG 7 Sequence-structure relationship of the C-terminal part of the BLV SU. (A) Bidimensional hydrophobic cluster analysis (HCA) plot of the BLV SU protein. The BLV SU protein is shown in duplicate where the hydrophobic amino acids (V, I, L, F, M, Y, and W) are countered (59). These residues form clusters that correspond to the internal faces of regular secondary structures (α-helices and β-strands) (60). Proline residues are shown as black stars, glycine residues are shown as black diamonds, threonine residues are shown as empty squares, and serine residues are shown as squares containing a black dot in the center. The regular secondary structures and variable regions of the N-terminal portion of the protein were deduced by comparison with the SU protein of the murine virus Fr-MLV, whose 3D structure has been solved (61). The predicted β-strands of the C-terminal part are shown in gray. (B) Multiple alignment of the C-terminal domain (CTD) sequences of surface units from different oncoviruses and related endogenous viruses. This sequence alignment was made using anchor points defined using iterative PSI-BLAST searches, as well as hydrophobic cluster analysis (62, 63), which allowed us to detect secondary structure conservation. Secondary structure predictions, reported above the alignment, were deduced from a combined hydrophobic cluster (62) and PSI-PRED (64) analysis. Secondary structures which were predicted to participate in the CTD core (conserved in all of the sequences) are colored black, whereas those which appear to be BLV specific (or BLV-HTLV specific) are indicated in gray. A proline-rich region (not shown) separates the CTD from the N-terminal receptor-binding domain (RBD). The following color code is used to indicate amino acid conservation—green, hydrophobic; orange, aromatic; blue, basic; pink, S and T; brown, P, G, D, N, and S; yellow, C. Potential N-glycosylation sites are highlighted in dark orange, N230 being highlighted with a red star. The UniProt identifier codes and accession numbers, as well as the amino acid (aa) CTD limits (within parentheses), were as follows: bovine leukemia virus, ENV_BLV P51519 (aa 186 to 301); human T-cell leukemia virus 1A, ENV_HTL1A P03381 (aa 195 to 312); human T-cell leukemia virus 2, ENV_HTLV2 P03383 (aa 191 to 308); Friend murine leukemia virus, ENV_MLVFR P03395 (aa 289 to 445); mink cell focus-forming murine leukemia virus and feline leukemia virus, ENV_MCFF P15073 (aa 284 to 440) and ENV_FLVCA Q02076 (aa 249 to 405); HERV-F(c)1_Xq21.33 provirus, EFC1_HUMAN P60507 (aa 229 to 383); HERV-F(c)2_7q36.2 provirus, EFC2_HUMAN P60608 (aa 257 to 410); and syncytin 1, SYCY1_HUMAN Q9UQF0 (aa 163 to 317).

the infectious cycle. Our preliminary data using inverted PCR nevertheless indicate that N230E infection is as polyclonal as wild-type BLV.

It is particularly noteworthy that, with shortened latency periods as observed in immunosuppressed splenectomized sheep (30), there is a trend to higher pathogenicity of N230E compared to the wild type. In fact, none of the previously characterized mutants exhibited this phenotype. For example, a mutant with optimal CREB-binding sites within the LTR is, as expected, better transcribed but replicates very inefficiently (57). A D247G mutation of Tax improves transcriptional activity but is neutral for replication *in vivo* (58). To our knowledge, N230E is the only rare mutant identified thus far that is more replication competent and pathogenic than the wild-type virus. Since the primary goal of a virus is to persist and replicate, why is mutant N230E not found in any reported BLV isolate? First, mutant N230E may not replicate faster in cattle as observed in sheep. Although unlikely, it is still possible that, in this particular species, a better neutralization of glycan-devoid epitopes limits replication despite an ongoing cell-to-cell infection. Second, N230E may impair natural BLV transmission through insects or milk, only wild-type viruses being able to cross this bottleneck, thereby restricting sequence divergence. Third, it is possible that this type of mutation occurs in naturally infected animals but was not previously identified. Ongoing deep-sequencing experiments are currently screening for the emergence of N230E quasipecies in chronically infected animals.

Among different hypotheses, we favor a model postulating that a pathogen and its host should reach an equilibrium allowing for the coexistence of both species. Replication and pathogenicity would impair persistence but potentially increase transmission between animals. Because BLV is poorly infectious, viral persistence without significant pathogenicity is essential for its persistence. We cannot, however, exclude that N230E mutation neither occurred in evolution nor will ever emerge as a hyperpathogenic strain.

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